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Prognostic value of BRAF V600E mutation and microsatellite instability in Japanese patients with sporadic colorectal cancer

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ABSTRACT

Purpose

In colorectal cancer (CRC), the BRAF V600E mutation is an important biomarker for poor prognosis, while high microsatellite instability (MSI-H) indicates good prognosis. Using a commercial BRAF V600E-specific antibody, we investigated the BRAF V600E mutation according to immunohistochemistry (IHC) and the MSI status in Japanese patients with CRC.

Methods

In this retrospective study, tissue samples from 472 Japanese patients with CRC, stratified for MSI, were analyzed to determine the prognostic value of BRAF V600E, as assessed using IHC. Mutations in 254 patients were evaluated using the direct sequencing method to check for concordance.

Results

The frequency of MSI-H was 9.3% (44/472), and BRAF V600E mutation was detected immunohistochemically in 8.7% patients (41/472). The sensitivity and specificity for detection of BRAF V600E mutations by IHC were 100% (17/17) and 98.7% (234/237), respectively. BRAF V600E mutations were significantly correlated with the anatomical tumor site ($P = 0.0035$), histologic type ($P < 0.0001$), and MSI status ($P < 0.0001$). Consistent with other published series, patients with BRAF V600E mutation exhibited a significantly shorter overall survival (hazard ratio = 1.500, $P = 0.0432$). In particular, the microsatellite stable/BRAF mutation group had inferior prognosis compared with the MSI-H/BRAF wild-type group (hazard ratio = 2.621, $P = 0.0004$).

Conclusions

IHC using a BRAF V600E-specific antibody was useful for diagnosis and concurred with direct sequencing results. CRC cases could be stratified by combining BRAF V600E mutation and MSI status as a prognostic factor in Japanese patients.

Keywords: Colorectal cancer, BRAF mutation, Microsatellite instability, Immunohistochemistry, Japanese patients

Introduction

Microsatellite instability (MSI) and B-type Raf kinase (BRAF) mutation status are significant prognostic fators in intrinsic colorectal cancer (CRC) subtypes (Bosman and Yan 2014; Jass 2007; Lochhead et al. 2013; Ogura et al. 2014; Phipps et al. 2015; Stachler et al. 2015). Therefore, the classification of intrinsic CRC subtypes has gained increasing attention for treatment strategy planning.

BRAF mutations are detected mainly via the activated domain within the kinase domain or via the ATP-binding site. The BRAF V600E missense mutation, which is a valine (V) to glutamic acid (E) switch at codon 600 caused by the c.1799T>A transversion, accounts for 80% of BRAF mutations. BRAF mutation frequencies in gastrointestinal cancers have been reported as 14% in CRC (Zheng et al. 2015), 13% in biliary cancer (Goeppert et al. 2014), 0% in liver cancer (Colombino et al. 2012), 7.1% in pancreatic cancer (Ishimura et al. 2003), 1% in small intestine cancer (Blaker et al. 2004), 1% in esophageal cancer (Maeng et al. 2012), and 1% in gastric cancer (Davies et al. 2002; Wu et al. 2004). The BRAF V600E mutation in CRC is associated with high kinase activity, with BRAF and KRAS mutations being mutually exclusive.

Microsatellite instability (MSI) is caused by a lack of or alteration in mismatch repair genes, which causes alterations in repetitive sequence length in the microsatellite DNA domain within neoplastic cells (Boland and Goel 2010; Yamamoto and Imai 2015). During classification of intrinsic subtypes in CRC, the BRAF mutation and high MSI (MSI-H) are sometimes grouped into the same category (Kambara et al. 2004; Rajagopalan et al. 2002) because MSI-H tumors often contain a BRAF mutation (Lochhead et al. 2013). One exception is Lynch syndrome (Moreira et al. 2012; Thiel et al. 2013), which is caused by 1 of 4 genetic germ cell line mutations in MLH1, MSH2, MSH6, PMS2, where 90% cases also show MSI alone. In MSI-H CRC, genome-wide sporadic methylation occurs in many genes such as MLH1 (Yamamoto and Imai 2015). Previous clinical trials have reported the BRAF gene mutation frequency and MSI status in CRC, but the concordance rates of BRAF mutations and MSI, as well as, their influence on CRC prognosis have not been explored. In the present study, to clarify the prognostic impact of combined BRAF and MSI status,

we investigated the relationship between the BRAF V600E mutation status, determined by IHC using a BRAF V600E mutation-specific antibody, and MSI status in 472 consecutive Japanese patients with CRC who presented at a single institution.

Materials and methods

Patients and specimens

We analyzed tissue samples from 472 Japanese patients with CRC who underwent surgical resection at the Department of Surgery and Science, Kyushu University Hospital, Japan, between 1994 and 2013. Histological diagnoses were based on the World Health Organization criteria. Pathological staging was performed according to the Tumor-Node-Metastasis classification system, revised in 2002 (Wittekind et al. 2002). Informed consent was obtained from each patient before tissue acquisition.

MSI analysis

MSI status was assessed using fluorescent-labeled primers and an automated DNA sequencer as described previously (Toh et al. 1996) (Oda et al. 1997) (Oki et al. 1999). Briefly, we amplified the microsatellite domain by polymerase chain reaction (PCR) from cancerous and normal tissues. The fluorescent-labeled PCR product was loaded on an ABI 310 sequencer and data were analyzed using Gene Scan software. MSI was determined according to the positive frequencies of 5 reference markers (D2S123, D5S107, D10S197, D11S904, and D13S175). MSI-H was defined as a replication error in ≥2 markers. Low MSI (MSI-L) was defined as a replication error in a single marker. MSS was defined as no replication error in these markers. MSS is considered as MSS and MSL-L (stable [MSS])/low [MSI-L] vs. MSI-H).

BRAF mutation analysis

BRAF mutations were mainly analyzed by IHC using a BRAF V600E-mutation specific antibody (Kuan et al. 2014). We also performed direct sequencing in 254 patient samples to examine the concordance of IHC with the actual gene mutation status. Direct sequencing could not be performed in all 472 cases because of insufficient DNA extraction from the formalin-fixed paraffin-embedded sections.

IHC staining using the VE1 mouse monoclonal antibody (Spring Biosciences, CA) was performed on 4-μm thick, formalin-fixed, paraffin-embedded tissue sections. The manual staining method was used to evaluate the factors affecting staining results including the pH of heat-induced antigen retrieval solutions and antibody concentrations. Briefly, 4-μm paraffin sections mounted on negatively charged glass slides were deparaffinized in xylene solution and rehydrated in a graded methanol series followed by washing in

distilled water. Antigen retrieval in 1 mmol/L ethylenediaminetetraacetic acid buffer (pH 8.0) was performed in a pressure cooker at 125°C for 15 minutes. The sections were allowed to cool and were washed with phosphate-buffered saline (PBS) containing 0.05% Tween 20 at pH 7.0. The sections were incubated in the VE1 antibody overnight in a humidified chamber at 20°C (1:50 dilution). After washing with PBS, the sections were incubated with horseradish peroxidase-labeled polymer antimouse secondary antibody (DAKO Envision+ system, Carpinteria, CA) for 1 hour at 20°C. Staining was developed with liquid DAB chromogen in imidazole-HCl buffer (pH 7.5) containing hydrogen peroxide until the brown color was developed fully. The sections were counterstained with hematoxylin, and a coverslip was placed using permanent mounting medium. For all cases, VE1 IHC slides were read independently as positive or negative, and readers were blinded to the PCR or BRAF mutation status data. For the retrospective series, each case was reviewed by a pathologist. A semiquantitative assessment of intensity was performed, as follows: 0, negative with no cytoplasmic staining at any magnification; 1+, weak, difficult to recognize, brown staining requiring ≥10× magnification for confirmation; 2+, moderate, easy to recognize, brown staining seen at 2× or 4× magnification; and 3+, strong staining, visible with the naked eye and at 2× magnification. Sections with a score of 1–3 were considered BRAF V600E mutation-positive. We analyzed BRAF V600E mutation status using the direct sequencing method in 254 specimens as described previously. Briefly, the BRAF region was amplified by PCR using the primers BRAF forward 5′-GCTTGCTCTGATACCAAAATGAG-3′ and reverse 5′-CCACAAAATGGATCCAGACA-3′ and Taq polymerase with 3-exonuclease activity (TaKaRa Ex Taq; Takara Bio Inc.). Purified PCR products were used as a template for the cycle sequencing kit (Applied Biosystems, Foster City, CA, USA).

Statistical analyses

Relationships between the clinicopathological factors, BRAF mutations, and MSI status were analyzed using the χ^2 test. Survival curves were plotted using the Kaplan–Meier method and the log-rank test was used to determine associations between individual variables and survival. Differences were considered significant at $P < 0.05$. We performed statistical analyses using JMP ver10. software.

Results

MSI status of all cases

The total frequency of MSI-H was 9.3% (44/472 cases). Of those with MSI-H, 11.5% (27/235 cases) had Stage I/II CRC and 7.2% (17/237 cases) had Stage III/ CRC. The clinicopathologic features according to MSI status are summarized in Table 1. There were significant differences in the clinicopathological features according to microsatellite stability (stable [MSS])/low [MSI-L] vs. MSI-H) as follows: poorly differentiated adenocarcinomas, 6.7% (29/428 cases) vs. 38.6% (17/44 cases); right-side tumors, 47.1% (202/428 cases) vs. 81.8% (36/44 cases); lymph node metastases, 49.3% (211/428 cases) vs. 25.0% (11/44 cases); BRAF mutation, 5.4% (23/428 cases) vs. 40.9% (18/44 cases) ($P < 0.05$). In addition, in the MSI-H group, a trend was noted favoring females 52.3% (23/44 cases) ($P = 0.083$).

BRAF V600E mutation

Representative BRAF V600E semiquantitative IHC is shown in Figure 1. Among the 472 cases analyzed by BRAF V600E IHC, BRAF gene mutation by direct sequencing was performed in 254 cases. According to BRAF direct sequencing 17/254 (6.7%) cases were BRAF V600E positive (Table 2). All 17 BRAF V600E mutation-positive cases detected by direct sequencing were positive according to IHC. BRAF V600E mutation detection by IHC had a sensitivity and specificity of 100% (17/17 cases) and 98.7% (234/237 cases), respectively, based on the results from direct sequencing. Table 3 shows a summary of clinicopathological features of BRAF V600E mutation according to IHC. Significant differences were noted in the BRAF V600E mutation-positive group compared with the non-mutated group as follows: poorly differentiated adenocarcinoma, 43.9% (18/41 cases), right side 75.6% (31/41 cases), MSI-H 43.9% (18/41 cases) ($P < 0.05$). In addition, in the BRAF V600E mutation-positive group, there was a tendency toward lymphatic invasion 51.2% (21/41 cases).

We examined 26 representative biopsy samples (Supplementary Table 1, Supplementary Figure 1). There was concordance in BRAF V600E mutation-positivity between resected specimens and biopsy specimens

in 8/9 cases. In 1 resected specimen positive for BRAF V600E mutation, the corresponding biopsy tissue was negative. All 17 cases judged to be BRAF V600E mutation negative in resected specimens were also negative in the biopsy samples. For BRAF V600E IHC in the biopsy specimens, the sensitivity was 88.9% (8/9 cases), and the specificity was 100% (17/17 cases).

Influence on prognosis of MSI status and BRAF V600E mutation

There were no significant differences in survival across all disease Stages according to MSI status (P = 0.4429; hazard ratio [HR], 1.423) (Figure 2). However, the BRAF V600E mutation-positive group (N = 41) had significantly inferior survival compared with the BRAF wild-type group (N = 431, P = 0.0432; HR, 1.500) (Figure 3). Regarding the relationship between MSI and BRAF mutation status, in the MSS group (N = 428), those with a BRAF mutation (N = 23) had a significantly inferior prognosis compared with that in those with BRAF wild-type (N = 405, P = 0.0004; HR, 2.621; Figure 4). However, in the MSI-H group (N = 44), there were no survival differences between the BRAF V600E mutation and BRAF wild-type groups (P = 0.4655, HR = 0.6443, Figure 5).

Discussion

For adequate treatment planning, it is now a requirement that the BRAF mutation and MSI status of patients with CRC are determined. Several studies reported that, after radical surgery, patients with MSI-H had a good prognosis, whereas those with a BRAF mutation had a poor prognosis (Garnett and Marais 2004; Lochhead et al. 2013; Nakanishi et al. 2013). By contrast, others have found no prognostic significance of BRAF mutations in patients with CRC (Barault et al. 2008). BRAF mutations and MSI-H are strongly related because of their influence on genome-wide CpG island methylation (Curtin et al. 2011; Wu and Bekaii-Saab 2012). Therefore, to predict CRC prognosis, it has been suggested that both BRAF mutation status and MSI status should be determined (Stachler et al. 2015).

The frequency of MSI-H in the radical surgery cases at our facility was 9.3%, which was lower compared with frequencies previously reported in European and North American studies (15–20%) (Lochhead et al. 2013; Phipps et al. 2015), but similar to that reported in Asian populations (Qiu et al. 2015). Consistent with previous reports, in the present study, the incidence of MSI-H cases was notably higher in patients with poorly differentiated adenocarcinoma and right-sided tumors; however, the incidence of MSI-H cases was notably lower in patients with lymph node metastasis. In addition, BRAF V600E mutations were significantly more prevalent in cases of poorly differentiated adenocarcinoma, right-sided tumors, and MSI-H, which was consistent with previous studies (Lochhead et al. 2013) (Toon et al. 2014) (Roth et al. 2010).

The IHC method of mutation detection has advantages in that, compared with direct sequencing, it is simple, quick, and inexpensive to perform. Firstly, IHC facilitates BRAF mutation detection without the need for DNA extraction, which can be time-consuming and challenging if there is insufficient tissue or if the tissue quality is poor, which can be the case with paraffin-embedded tissues that have been stored for extensive periods. In addition, non-cancerous tissues are often mixed with cancer tissues; therefore, DNA extracted would come from both tissue sources, which could cause false-positive or false-negative results. In a previous study in 477 patients with CRC (Day et al. 2015), BRAF V600E mutation status assessed by IHC and the Sanger sequencing method using a tissue microarray were compared. In that study, compared with the sequencing method, IHC had a sensitivity and specificity of 98.2% and 98.1%,

respectively. In the present study, BRAF V600E mutations detected by sequencing were confirmed by IHC with a sensitivity and specificity of 100% and 98.7%, respectively. In a study of 611 Chinese patients with CRC (Qiu et al. 2015), BRAF V600E IHC and Sanger sequencing results were compared in 181 cases. In that study, the sensitivity and specificity of IHC were 100% and 99%, respectively, which were similar to the values obtained in the present study. Therefore, where IHC facilities are available, BRAF V600E IHC could be useful for determining mutational status in the diagnosis of CRC.

Patients with BRAF mutations tend to have a poor prognosis (Lochhead et al. 2013). In the present study, patients with BRAF mutations had significantly inferior survival rates compared to those without mutations. Interestingly, in patients with MSS, the prognosis of patients with the BRAF mutation was poorer than that in those with BRAF wild-type. By contrast, survival in the MSI-H group was not affected by the BRAF genotype. This suggests that survival was only affected by BRAF status in patients with stable microsatellites, which further emphasizes the need to evaluate both BRAF mutation and MSI status in patients with CRC for an accurate prognosis. In a previous study, the MSI-H/BRAF mutation group also had a good prognosis (Lochhead et al. 2013). In CRC patients with sporadic MSI-H, radical resection was associated with a good prognosis, but recurrence was associated with a poor prognosis (Boland and Goel 2010). Furthermore, BRAF mutation status did not influence the prognosis in similar cases (French et al. 2008; Phipps et al. 2015; Roth et al. 2010; Wu and Bekaii-Saab 2012). The present study is the first IHC analysis to show the same associations; however, further studies investigating the lack of effect of BRAF mutation on survival in patients with MSI-H are needed to validate these findings. Recently, FOLFOXIRI plus bevacizumab chemotherapy or BRAF inhibitors have been considered for the treatment of CRC patients with BRAF mutations (Loupakis et al. 2014; Yan and Grothey 2015). However, in future, it might be necessary to stratify patients with BRAF mutation according to the MSI status to determine the prognosis and most appropriate treatment regimen. We investigated prognosis in patients in the MSS/BRAF V600E mutation group who did or did not undergo adjuvant chemotherapy (Supplementary Figures 2, 3), but there were no significant differences in survival between them. Adjuvant chemotherapy efficacy in patients with MSS is good when compared with that in those in MSI-H group; however, based on the poorer prognosis, more intensive adjuvant chemotherapy might be necessary for those with stable microsatellites who have concomitant BRAF mutations.

In the present study, MSI and BRAF mutation status were important genetic factors that facilitate the initial

planning of individualized treatment strategies for Japanese patients with CRC, which could improve prognosis in those with more aggressive genetic variants of CRC.

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Compliance with ethical standards

Conflict of interest statement

All authors declare no conflicts of interest with regard to this study.

Human and animal rights

All procedures performed in studies involving human participants were conducted in accordance with the ethical standards of the Institutional and National Research Committees and with the 1964 Helsinki Declaration and its later amendments.

Informed consent

Informed consent was obtained from all participants included in the study.

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